Immunogenicity of Recombinant Attenuated *Salmonella enterica* Serovar Typhimurium Vaccine Strains Carrying a Gene That Encodes *Eimeria tenella* Antigen SO7[▽]

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Recombinant attenuated *Salmonella* vaccines against avian coccidiosis were developed to deliver *Eimeria* species antigens to the lymphoid tissues of chickens via the type 3 secretion system (T3SS) and the type 2 secretion system (T2SS) of *Salmonella*. For antigen delivery via the T3SS, the *Eimeria tenella* gene encoding sporozoite antigen SO7 was cloned downstream of the translocation domain of the *Salmonella enterica* serovar Typhimurium *sopE* gene in the parental pYA3868 and pYA3870 vectors to generate pYA4156 and pYA4157. Newly constructed T3SS vectors were introduced into host strain χ8879 (Δ*phoP233* Δ*sptP1033::xylE* Δ*asdA16*), an attenuated derivative of the highly virulent UK-1 strain. The SopE-SO7 fusion protein was secreted by the T3SS of *Salmonella*. The vector pYA4184 was constructed for delivery of the SO7 antigen via the T2SS. The SO7 protein was toxic to *Salmonella* when larger amounts were synthesized; thus, the synthesis of this protein was placed under the control of the *lacI* repressor gene, whose expression in turn was dependent on the amount of available arabinose in the medium. The pYA4184 vector was introduced into host strain χ9242 (Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P$_{BAD}$ *lacI* TT [TT is the T4*ipIII* transcription terminator]). In addition to SO7, for immunization and challenge studies we used the EAMZ250 antigen of *Eimeria acervulina*, which was previously shown to confer partial protection against *E. acervulina* challenge when it was delivered via the T3SS. Immunization of chickens with a combination of the SO7 and EAMZ250 antigens delivered via the T3SS induced superior protection against challenge by *E. acervulina*. In contrast, chickens immunized with SO7 that was delivered via the T2SS of *Salmonella* were better protected from challenge by *E. tenella*.

Eimeria spp. are intestinal protozoan parasites and causative agents of coccidiosis, a ubiquitous disease of poultry that is characterized by intestinal lesions, blood loss, reduced weight gain and feed efficiency, increased susceptibility to other disease agents, and, in severe cases, mortality (1, 38, 40). For over 50 years coccidiosis has been controlled mainly by prophylactic chemotherapy. During this time, the emergence of drug-resistant *Eimeria* strains, the high cost of new drug development, and increased public opposition to the inclusion of chemicals in animal feed have led to a shift in the approach to development of vaccines against this parasite (4, 14, 16). Except for a very short period, the life cycle of *Eimeria* spp. occurs within the intestinal epithelium; thus, induction of *Eimeria*-specific immune responses in underlying mucosal tissues is critically important for protection against this disease. *Eimeria* is an intracellular parasite, and cell-mediated immunity, mediated mainly by antigen-specific and nonspecific activation of macrophages and T lymphocytes, plays a major role in protection (1, 24). A hallmark of the immune response to *Eimeria* infection appears to be the development of Th1-type, gamma interferon-mediated immunity with natural killer cells, cytotoxic CD8$^+$ T cells, and helper CD4$^+$ T cells (as well as cytokines

secreted by these cells) at the mucosal site of infection (gut) (1, 24). During infection, antibodies specific to *Eimeria* are generated and might be important for binding and neutralizing *Eimeria* while it is in the extracellular stages of development; however, the importance of these antibodies in protection against *Eimeria* has been questioned (23, 24).

Exposure of chicks to one species of *Eimeria* generates protective immunity against the homologous species. However, chickens remain susceptible to infection by one of the other six species of *Eimeria*. Live attenuated *Eimeria* vaccine strains, although effective, are costly to produce and have a limited shelf life (4). In comparison, recombinant attenuated *Salmonella* vaccine (RASV) strains, which are able to harbor and deliver heterologous antigens, have several advantages. In addition to being safe, easy to administer, and affordable, they colonize the mucosa and other lymphoid tissues for prolonged periods of time and induce mucosal and systemic immune responses, both humoral and cell mediated (7, 17, 20, 34). RASV strains have been used to immunize chicks with two *Eimeria acervulina* antigens, which resulted in induction of cell-mediated immune responses and protective immunity against *E. acervulina* challenge infection (20). In order to diversify the repertoire of the induced immune responses, we have used two antigen delivery approaches: the type 3 secretion system (T3SS) and the type 2 secretion system (T2SS) of *Salmonella*. The T3SS, a needle-shaped organelle of *Salmonella* consisting of over 20 structural or secreted proteins, is critically important during the intestinal phase of infection

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
E. coli strains		
χ6212	F$^-$ λ$^-$ φ80 Δ(*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*	26
χ7213	*thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44* Δ*asdA4 recA1*	29
S. enterica serovar Typhimurium strains		
χ3761	UK-1 wild type	7
χ8879	Δ*asdA16* Δ*phoP233* Δ*sptP1033*::*xylE*	20
χ9085	Δ*asdA16* Δ*phoP233* Δ*sptP1033*::*xylE hisG atrB13*::Mu*dJ*	20
χ9242	Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P$_{BAD}$ *lacI* TT	This study
Plasmids		
pYA3493	T2SS signal sequence-based periplasmic secretion plasmid	17
pYA3658	792 bp DNA encoding EAMZ250 in pYA3653	20
pYA3700	Cloning vector containing *araC* P$_{BAD}$ cassette	pYA3698
pYA3868	Asd$^+$; p15A*ori*; P$_{asd}$ *asd* P$_{sopE}$ SD-(GTG)-*sopE* (N-terminal region)	pYA3332
pYA3870	Asd$^+$; p15A*ori*; P$_{asd}$ *asd* P$_{sopE}$ SD-(ATG)-*sopE* (N-terminal region)	pYA3332
pYA4064	Suicide vector harboring *relA*::*araC* P$_{BAD}$ *lacI* TT	pRE112
pYA4156	740 bp DNA encoding the SO7 antigen in pYA3868	pYA3868
pYA4157	740 bp DNA encoding the SO7 antigen in pYA3870	pYA3870
pYA4184	740 bp DNA encoding the SO7 antigen in pYA3493	pYA3493

(10). The T3SS enables *Salmonella* to inject its effector proteins into the cytoplasm of the host cell and modulate its cellular functions and signal transduction pathways (10, 11). Two of the effector proteins, SptP and SopE, have been used as means by which RASV strains translocate heterologous antigens into the cytoplasm of host cells to induce antigen-specific cytotoxic T-lymphocyte responses (8, 20, 30, 35). A strong and persistent cytotoxic T-lymphocyte response to lymphocytic choriomeningitis virus in mice was observed when a protective major histocompatibility complex class I epitope fused to *Salmonella* effector protein SptP was delivered (35). This virus-specific immunity was sufficient to provide protection against challenge with lymphocytic choriomeningitis virus (35). Other workers (8) delivered fragments of the simian immunodeficiency virus Gag protein fused to *Salmonella* effector protein SopE and were able to induce virus-specific CD4$^+$ and CD8$^+$ T-cell responses in Rhesus macaques; however, this was not sufficient to protect against an intrarectal challenge with simian immunodeficiency virus mac239.

In contrast to the T3SS, the T2SS has been shown to induce Th1/Th2 immune responses with antibody titers to heterologous antigen that rival the antibody titers to lipopolysaccharide, a *Salmonella* antigen (17). A combination of antigen delivery strategies and use of multiple protective antigens could lead to generation of a more diverse immune response and possibly superior protection against challenge. EAMZ250 of *E. acervulina* (20) and the SO7 antigen of *Eimeria tenella* (6) were chosen for delivery via RASV strains. Previously, RASV strain-delivered EAMZ250 induced partial protection against *E. acervulina* challenge (20), while SO7, delivered using intramuscular injection, induced protection against challenge with four different species of *Eimeria* (6). The use of antigens specific to two different developmental stages, sporozoites (SO7) and merozoites (EAMZ250), may lead to more complete immunity against the parasite. Other workers have delivered the SO7 antigen via attenuated *Salmonella* strains and shown that

there was induction of antibody titers (immunoglobulin G [IgG] and IgA) to this antigen in immunized chickens, although no cell-mediated immune responses or *Eimeria* challenge studies were described (27).

The SO7 antigen, fused to the 80-amino-acid translocation domain of effector protein SopE, can be secreted via the T3SS of RASV strains. However, when larger amounts of SO7 are expressed by a medium-copy-number plasmid (pBR *ori*) in T2SS vectors, SO7 becomes toxic to *Salmonella*. To deliver this antigen without a detrimental effect on RASV strains, its expression was placed under control of the repressor gene *lacI*, whose expression in turn is dependent on the presence of arabinose in the medium. The lack of available arabinose in tissues removes the LacI repression of SO7 synthesis, enabling *Salmonella* to express this antigen in vivo during colonization of lymphoid tissues. For expression of the recombinant antigen, *Salmonella* vaccine strains with a deletion of the *asd* gene, which is essential for synthesis of the bacterial cell wall diaminopimelic acid (DAP), were used. The *asd* deletion in host strains is complemented with antigen-bearing Asd$^+$ plasmids (26).

The results of this study indicate that RASV strains expressing *Eimeria* antigens are able to colonize and persist in tissues of 1-week-old chickens with an established microflora, resulting in induction of antigen-specific antibody titers and protective immune responses to challenge with *E. acervulina* or *E. tenella* oocysts.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriophage P22HT*int* (33) was used for generalized transduction. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium cultures were grown at 37°C in Luria-Bertani (LB) broth, in Lennox broth (22), or on LB agar (2). MacConkey agar (Difco, Detroit, MI) supplemented with 1% lactose was used for fermentation assays. When required, antibiotics were added to culture media at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50
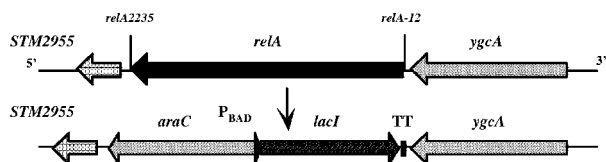
Deletion of 2247 bp (*relA-12* to *relA2235/2235*) and insertion of 2429 bp of *araC* P$_{BAD}$ *lacI*

Δ*relA198*::*araC* P$_{BAD}$ <u>AGGA</u>TGGTGAAT ATG-*lacI TT**
SD

FIG. 1. (A) Chromosomal deletion map for recombinant attenuated *Salmonella* vaccine strain χ9242 (Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P$_{BAD}$ *lacI* TT). The Shine-Delgarno (SD) sequence was improved from AGGG to AGGA, the start codon was improved from GTG to ATG, and *lacI* codons were optimized for a high level of expression in *Salmonella*.

μg/ml; and tetracycline, 15 μg/ml. DAP (50 μg/ml) was added for growth of Asd⁻ strains (26). LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in allelic exchange experiments (12).

General DNA procedures. DNA manipulations were performed using standard procedures (31). *E. coli* and *S. enterica* serovar Typhimurium were transformed by electroporation (Bio-Rad, Hercules, CA). Transformants containing Asd⁺ plasmids were selected on LB agar plates without DAP. Recombinant suicide plasmids were transferred to *Salmonella* by conjugation using *E. coli* χ7213 (29) as the plasmid donor. Bacteriophage P22HT*int*-mediated general transduction was performed by standard methods (37). Constitutive expression of β-galactosidase in RASV strain χ8879 was made possible by introducing the *atrB13*::Mu*d* J allele by P22-mediated transduction from χ4574, resulting in strain χ9085 (9, 20). PCR amplification was employed to obtain DNA fragments for cloning and for verification of chromosomal deletion mutations. Nucleotide sequencing reactions were performed using ABI Prism fluorescent BigDye terminators according to the instructions of the manufacturer (PE Biosystems, Norwalk, CT).

Construction of χ9242. Phage P22 transduction was used to introduce the *araBAD23* mutation into *S. enterica* serovar Typhimurium χ8916 to generate χ9098. Construction of the pYA4064 suicide vector that was used to construct vaccine strain χ9242 has been described previously (S. Wang, Y. Li, G. Scarpellini, W. Kong, and R. Curtiss III, unpublished data). Δ*relA198*::*araC* P$_{BAD}$ *lacI* TT (TT is the T4*ipIII* transcription terminator) was introduced into χ9098 by allelic exchange using the suicide vector pYA4064 to obtain χ9242 (Fig. 1). The presence of the 3.3-kb deletion-insertion was confirmed by PCR with forward primer *RelA* N-HindIII (5′-CCC<u>AAGCTT</u>GAGCTCGAGGGCGTTCCGGCG CTGGTAGAA-3′ [HindIII site underlined]) and reverse primer *relA* C-KpnI (5′-CG<u>GGTACCC</u>CCAGATATTTTCCAGATCTTCAC-3′ [KpnI site underlined]).

Characterization of phenotype. The phenotype of vaccine strain χ8879 was confirmed as described previously (20). The presence of the *araBAD23* mutation in χ9098 was verified by PCR and by demonstrating that this strain had a white colony phenotype on MacConkey agar plates supplemented with 1% arabinose. To detect RASV strains in tissue samples, we used MacConkey agar supplemented with 1% lactose. In addition, χ8879 colonies were distinguished from χ9242 colonies from tissue homogenates by spraying 0.25% catechol (Sigma) onto colonies on LB agar. The presence of the *asdA16* mutation in RASV strains was confirmed by PCR and by the inability of the strains to grow on media without DAP (26). The lipopolysaccharide profiles of RASV strains were examined by using previously described methods (13).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses. The expression of protein antigens was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting as described previously (20). The following antibodies were used: monoclonal mouse anti-hemagglutinin (HA) or mouse anti-β-galactosidase antibodies (Sigma), followed by alkaline phosphatase (AP)-conjugated goat anti-mouse or goat anti-rabbit IgG (Southern Biotech, Birmingham, AL). Immunoreactive bands were detected by addition of nitroblue tetrazolium—5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma).

Construction of vectors for antigen delivery via the T3SS. *E. coli* χ6212 was used as an intermediate host strain for cloning. The Asd⁺ vectors pYA3868 and pYA3870 with the p15A origin of replication were used for delivering antigens via the T3SS of *Salmonella*.

The *SO7* gene of *E. tenella* (28) was amplified by PCR with forward primer 5′-ATA<u>GAATTC</u>CTCGCCCCAACTTTTTCCCCC-3′ (EcoRI site underlined) and reverse primer 5′-TATAT<u>GTCGAC</u>ATTAAGCGTAGTCTGGGACGTC G-3′ (SalI site underlined) and cloned into pYA3868 and pYA3870 fused to the truncated *sopE* gene (encoding the 80-amino-acid translocation domain) (21) to generate plasmids pYA4156 and pYA4157, respectively. The gene encoding the HA epitope YPYDVPDYA (HA tag) from influenza virus A was added by PCR to the N-terminal region of the *SO7* gene in order to facilitate its detection.

Construction of vectors for antigen delivery via the T2SS. For antigen delivery by the T2SS, the *SO7* gene was PCR amplified with forward primer 5′-ATA<u>G AATTC</u>CTCGCCCCAACTTTTTCCCCC-3′ (EcoRI site underlined) and reverse primer 5′-TATAT<u>GTCGAC</u>ATTAAGCGTAGTCTGGGACGTCG-3′ (SalI site underlined) and cloned into pYA3493 to obtain vector pYA4184. The HA tag was added by PCR to the N-terminal region of the *SO7* gene. In-frame cloning of the *SO7* gene was confirmed by nucleotide sequencing. During cloning of *SO7* into pYA3493 we encountered difficulties stemming from the toxicity of SO7 for *Salmonella* cells. Thus, the SO7-encoding vector was transferred into χ6212 harboring pYA232, and the synthesis of the SO7 protein was analyzed with and without induction of gene expression by isopropyl-β-D-thiogalactopyranoside (IPTG). Following expression studies, newly constructed RASV strain χ9242 was transformed with pYA4184, in which the synthesis of the SO7 antigen was regulated by supplemental arabinose in the medium.

Determination of plasmid stability. Plasmid stability for approximately 50 generations of growth under permissive conditions was determined as described previously (20).

Secretion of SO7 into the culture supernatant. The secretion of SO7 antigen by χ9085 harboring pYA4156 or its parental precursor pYA3868 or harboring pYA4157 or its parental precursor pYA3870 was analyzed as described previously (18, 20). Secreted SopE-SO7 fusion protein was detected with mouse anti-HA monoclonal antibodies coupled to AP. To determine that the antigen was secreted actively via the T3SS rather than released into the culture supernatant due to cell lysis, supernatant and pellet samples of χ9085 were analyzed by immunoblotting to determine the presence of the cytoplasmic marker β-galactosidase using anti-β-galactosidase antibodies (Sigma).

Chickens. Outbred male chickens (Sexsal; Moyers Hatchery, Quakertown, PA) were reared in Petersime starter cages (Petersime Incubator Co., Gettysburg, OH) with feed and water provided ad libitum.

Animal infectivity. Vaccine strains were grown overnight in 20 ml of LB broth at 37°C. Strain χ9242 was grown in LB broth supplemented with 0.2% arabinose. The following day, 100 ml of LB broth (with or without supplemental arabinose) was inoculated with an overnight culture (1:100) and grown with aeration (180 rpm) in a 250-ml flask at 37°C until the optical density at 600 nm was 0.9 to 0.95. Cultures were centrifuged at room temperature (7,000 × *g* for 15 min), and each pellet was resuspended in 1 ml of buffered saline with gelatin (BSG). The titers of RASV strains used to inoculate chickens were determined by plating dilutions of the RASV strains on MacConkey agar supplemented with 1% lactose. One-week-old chicks were fasted for approximately 10 h and then orally inoculated with either 50 μl of BSG or 50 μl of BSG containing 1 × 10⁹ CFU of an RASV strain. On days 3 and 6 after oral immunization, samples of the spleen, cecum, and bursa were collected from three chickens per immunization group and homogenized in 1 ml (total volume) of BSG, and 10⁻¹ to 10⁻⁶ dilutions (depending on the tissue) were plated on MacConkey agar to determine the numbers of viable bacteria. Samples that were negative for the presence of RASV strains were recorded as containing less than 10 CFU.

Humoral immune response to *Eimeria* antigen. In one study 1-week-old chickens were immunized with RASV strains harboring only the SO7 antigen. The immunization groups were as follows: group G1, unimmunized control (only 50 μl BSG); group G2, χ8879 harboring pYA3870 (vector control), followed by χ9242 harboring pYA3493 (vector control); group G3, χ8879 harboring pYA4157 (T3SS-SO7), followed by χ8879 harboring pYA4157 (T3SS-SO7); group G4, χ8879 harboring pYA4157 (T3SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7); group G5, χ9242 harboring pYA4184 (T2SS-SO7), followed by χ8879 harboring pYA4157 (T3SS-SO7); and group G6, χ9242 harboring pYA4184 (T2SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7). Five chickens from each group were bled via a brachial (wing) vein when they were 1 week old (unimmunized) and 5 weeks old (immunized). The sera were diluted (1:250) in phosphate-buffered saline containing 0.05% Tween 20 (PBS-TW) and applied to duplicate wells of microtiter enzyme-linked immunosorbent assay (ELISA) plates (Nunc Immulon II) coated with soluble *E. tenella* sporozoite antigen. The coating antigen was prepared by extracting with a mini bead beater 10⁷ *E. tenella* oocysts in aqueous buffer containing the protease inhibitor

phenylmethylsulfonyl fluoride (Sigma) and 200 mg 0.5-mm glass beads. Insoluble *E. tenella* oocyst proteins were pelleted by centrifugation for 15 min at 12,000 × *g* with a microcentrifuge. The supernatant containing soluble *E. tenella* protein was used to coat ELISA microtiter wells at a concentration equivalent to ∼10⁵ sporozoites/well. Soluble protein was adsorbed to the well surface for 1 h at 37°C and then overnight at 4°C. Unbound antigen was removed from the wells, and each plate was washed once with PBS-TW and then incubated with phosphate-buffered saline containing 2% fetal calf serum to block nonspecific binding of chicken antibodies in subsequent steps. After blocking, the ELISA plates were washed once with PBS-TW, and then individual sera from all groups were applied in duplicate to *E. tenella* antigen-coated wells. All subsequent incubation steps were performed at 37°C. After 2 h, the wells were washed three times with PBS-TW and then incubated for 1 h with a 1:100 dilution of rabbit anti-chicken IgG(H+L) specific (Sigma) and for 1 h with 1:500 dilution of AP-labeled sheep anti-rabbit IgG (Sigma). All wells were washed three times between steps with PBS-TW. Binding was measured after addition of the *p*-nitrophenyl phosphate substrate (1 mg/ml; Sigma) in AP buffer (pH 9.5). Color development was determined at 405 nm using a Bio-Tek EL312 microtiter plate reader (Bio-Tek Instruments).

Immunization of chickens with RASV strains and challenge with *E. acervulina* or *E. tenella* oocysts. Two challenge studies were performed. In both studies 1-day-old chickens were assigned randomly to appropriate groups with 21 chickens per group in starter chick cages. When they were 7 days old, chickens were inoculated orally with 10⁹ CFU of an RASV strain in 50 µl BSG. The second dose of RASV strains was administered 1 week later.

(i) Experiment 1. A combination of RASV strains harboring EAMZ250 or SO7 antigens were used for immunization. The immunization groups were as follows: group G1, unimmunized control (only 50 µl BSG); group G2, χ8879 harboring pYA3870 (T3SS-vector control), followed by χ9242 harboring pYA3493 (T2SS-vector control); group G3, χ8879 harboring pYA3658 (T3SS-EAMZ250), followed by χ8879 harboring pYA4157 (T3SS-SO7); group G4, χ8879 harboring pYA3658 (T3SS-EAMZ250), followed by χ9242 harboring pYA4184 (T2SS-SO7); group G5, χ8879 harboring pYA4157(T3SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7); and group G6, χ9242 harboring pYA4184 (T2SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7). Three weeks after the second immunization (when the animals were 5 weeks old), the chickens in groups G2 to G6 were orally challenged with 10⁵ *E. acervulina* oocysts.

(ii) Experiment 2. RASV strains harboring only the SO7 antigen were used. The immunization groups were as follows: group G1, unimmunized control (only 50 µl BSG); group G2, χ8879 harboring pYA3870 (T3SS-vector control), followed by χ9242 harboring pYA3493 (T2SS-vector control); group G3, χ8879 harboring pYA4157 (T3SS-SO7), followed by χ8879 harboring pYA4157 (T3SS-SO7); group G4, χ8879 harboring pYA4157 (T3SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7); and group G5, χ9242 harboring pYA4184 (T2SS-SO7), followed by χ9242 harboring pYA4184 (T2SS-SO7). Three weeks after the second immunization (when the animals were 5 weeks old), the chickens in groups G2 to G5 were orally challenged with 10⁵ *E. tenella* oocysts. To measure protection against oocyst challenge, body weights and feed consumption were recorded just before the oocyst challenge and 1 week later.

Statistical analysis. Data were analyzed by using the general linear model procedure of SAS software (32). Group means were separated by using Tukey's multiple-comparison procedure and were considered significantly different if the *P* value was <0.05. Data were expressed as means ± standard errors of the means.

RESULTS

Plasmid stability. The *asd* gene was used for stable maintenance of the antigen-bearing vectors in the vaccine strains and served as a selective determinant to complement the chromosomal Δ*asd* mutation (26). The T3SS vectors (parental plasmids pYA3868 and pYA3870 and their SO7 gene-bearing counterparts, plasmids pYA4156 and pYA4157) and the T2SS vectors (parental plasmid pYA3493 and the SO7 gene-bearing plasmid pYA4184) complemented the *asd* mutations of *S. enterica* serovar Typhimurium host strains χ8879 and χ9242 (Fig. 1), respectively. All the vectors mentioned above were stably maintained for 50 or more generations in their *S. enterica* serovar Typhimurium Asd⁻ host strains grown in the presence or absence of DAP (data not shown). Since it was found that

the SO7 antigen was toxic to *Salmonella* when larger amounts were expressed (as was the case with T2SS vector pYA4184), 10 colonies were randomly selected after χ9242 harboring pYA4184 was passaged for more than 50 generations in medium supplemented with DAP and examined for expression of the SO7 protein by Western blotting (data not shown). This was done to ensure that the *SO7* insert was retained stably in the pYA4184 plasmid. Stable plasmid maintenance by RASV strains is critically important for the ability to colonize and persist in lymphoid tissues in order to deliver antigen and induce antigen-specific immune responses. Therefore, we investigated the in vivo stability of Asd⁺ plasmids encoding *Eimeria* antigens EASZ240 and EAMZ250. Twenty-eight days after immunization, RASV strains χ8879(pYA3657) and χ8879(pYA3658) were isolated from tissues, and 40 randomly selected single colonies of each RASV strain were analyzed by restriction digestion for the presence of the *Eimeria EASZ240* and *EAMZ250* genes, respectively. We found that vectors isolated from all colonies contained either the *EASZ240* or *EAMZ250* gene. Furthermore, all 20 randomly selected colonies of χ8879(pYA3657) that were positive for the presence of *EASZ240* expressed the EASZ240 protein antigen (unpublished observations).

Expression of the SO7 protein by T3SS and T2SS vectors. Synthesis of the SopE-SO7 fusion was placed under the control of a *sopE* promoter (P*sopE*; yYA4156 and pYA4157). In addition, the amount of antigen synthesized from the vectors was determined by using either an ATG (pYA4157) or GTG (pYA4156) start codon for the *sopE* gene (Fig. 2A). All T3SS vectors specified synthesis of a 35.2-kDa SopE-SO7 fusion protein, as expected, and the pYA4157 vector [P*asd* *asd* P*sopE* SD-(ATG)-*sopE*] specified synthesis of the largest amount of SopE-SO7 (Fig. 2B). The expression of a 28-kDa SO7 protein by the T2SS vector pYA4184 (Fig. 3) was regulated by the presence of supplemental arabinose in the medium. When χ9242 harboring this vector was grown in nutrient broth (NB) supplemented with 0.2% arabinose, little or no synthesis of the SO7 protein was observed (Fig. 3), while the amount of SO7 synthesized was much larger when this strain was grown in arabinose-free NB (Fig. 3).

Secretion of fusion protein SopE-SO7 into the culture supernatant. The secretion of T3SS effector proteins by *Salmonella* is regulated by various environmental factors. In order to maximize secretion of SopE-SO7 via the T3SS, RASV strains were grown in media with high osmolarity (300 mM NaCl), a high pH (pH 8.2), and little aeration. When preparations were probed with anti-HA antibodies, the 35.2-kDa SopE-SO7 fusion protein was detected in cell pellets and culture supernatants of χ9085 harboring the antigen-encoding plasmids pYA4156 and pYA4157, while no protein that was a similar size was detected in pellets or culture supernatants of χ9085 harboring pYA3868 and pYA3870 (vector control groups) (Fig. 4A). β-Galactosidase, a cytoplasmic marker, was found only in the cell pellet fractions of all groups and was absent in the corresponding concentrated culture supernatants, indicating that SopE-SO7 was actively secreted via the T3SS and was not released due to membrane leakage and cell lysis (Fig. 4B).

Animal infectivity. RASV strains colonize the cecum, bursa, and spleen of specific-pathogen-free (SPF) chickens very efficiently (20). We sought to determine whether RASV strains
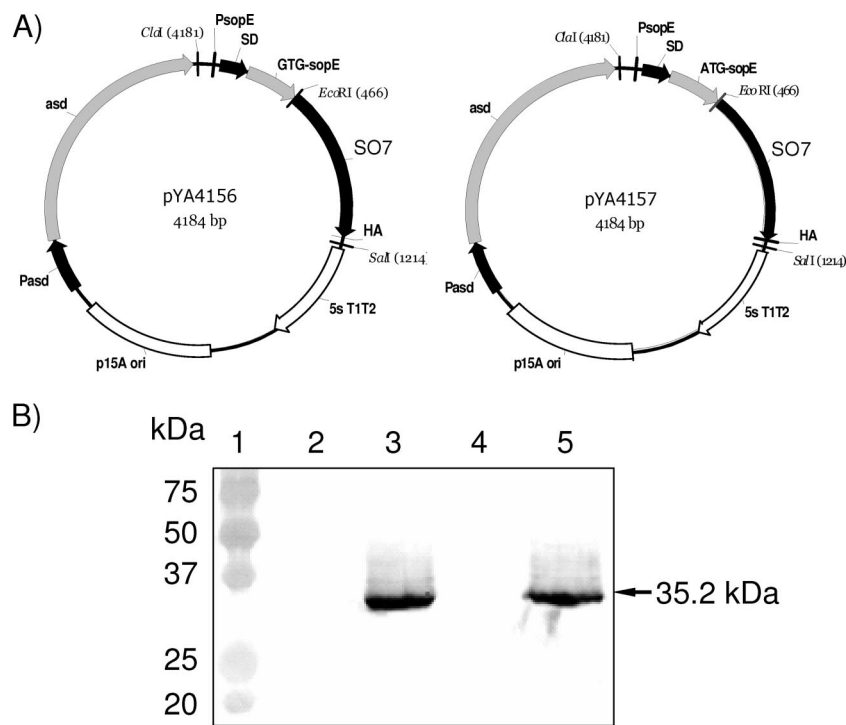
FIG. 2. (A) Vectors used for delivery of the *Eimeria* antigen SO7 via the T3SS. A 0.74-kb EcoRI-HindIII fragment of the PCR-amplified *SO7* gene was cloned into the EcoRI and HindIII sites of pYA3868 and pYA3870, respectively, yielding pYA4156 and pYA4157, to obtain the SopE-SO7 fusion. (B) Synthesis of the SopE-SO7 protein encoded by χ8879 harboring one of the two T3SS vectors was detected by Western blotting using primary mouse anti-HA monoclonal antibodies, followed by anti-mouse AP secondary antibodies. Lane 1, protein standard (Promega); lane 2, pYA3868; lane 3, pYA4156; lane 4, pYA3870; lane 5, pYA4157.

used to deliver the *E. tenella* antigen SO7 could successfully colonize tissues of non-SPF chickens that were 1 week old and had an established intestinal microflora which could potentially compete with the RASV strains for colonization of intestinal tissues. In addition, 1-week-old chicks commonly harbor maternally transmitted anti-*Salmonella* antibodies, which could hinder tissue colonization by RASV strains. Although *E. coli* was found in the intestine and bursa of all groups, no *Salmonella* colonies were obtained for tissues of unimmunized chicks, indicating that there was no tissue colonization by *Salmonella* spp. prior to immunization (Table 2). On day 3 after the primary immunization, the cecum, bursa, and spleen had high titers of the RASV strains (Table 2), although the titers were slightly lower than the titers observed previously for tissues of SPF chickens (20). Identical titers of RASV strains

were obtained for tissues of chickens collected on day 3 after the second immunization (data not shown).

Humoral immune responses in immunized chickens. When the animals were 1 week old, prior to RASV immunization, the
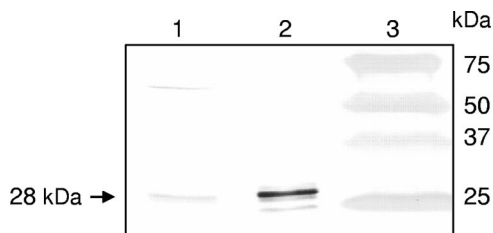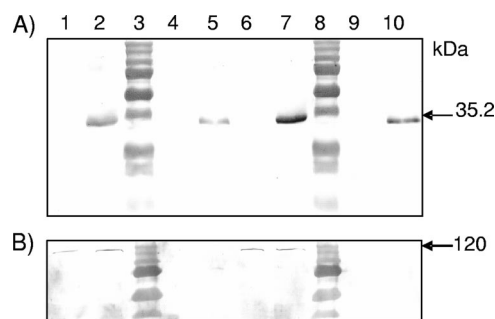

FIG. 4. (A) Secretion of SopE-SO7 into the culture supernatant. The RASV strains were grown with high osmolarity and a low level of aeration to simulate the conditions in the intestinal environment. Secreted SopE-SO7 fusion protein was detected by immunoblotting using anti-HA-AP monoclonal antibodies. Lanes 1, 4, 6, and 9, RASV strain χ9085 harboring vector control plasmids pYA3868 and pYA3870; lanes 2 and 7, cell pellet fraction of RASV strain χ9085 harboring pYA4156 (lane 2) or pYA4157 (lane 7); lanes 5 and 10, concentrated supernatant fraction of χ9085 harboring pYA4156 (lane 5) or pYA4157 (lane 10); lanes 3 and 8, protein standard (Promega). (B) β-Galactosidase was used as a cytoplasmic protein marker to detect whether the release of SO7 by χ9085 into the culture supernatant was due to cell lysis, and it was detected with anti-β-galactosidase antibodies. The contents of the lanes were the same as those described above for panel A.

FIG. 3. Synthesis of the SO7 protein in vaccine strain χ9242 grown in NB with or without 0.2% arabinose. Lane 1, NB with 0.2% arabinose; lane 2, NB without arabinose; lane 3, protein standard (Promega).

TABLE 2. Isolation of RASV strains from chicken tissues 3 days following the first inoculation

Vaccine strain	No. of organisms isolated from tissues 3 days after immunization[a]		
	Spleen	Bursa	Cecum
None (unimmunized)	0	0	0
χ8879(pYA3870) (T3SS-vector control)	1.9 ± 1.60 (1)[b]	5.6 ± 0.93	7.2 ± 0.56
χ8879(pYA4157) (T3SS-SO7)	0.7 ± 1.21 (2)	5.4 ± 1.06	6.0 ± 0.50
χ9242(pYA4184) (T2SS-SO7)	0.9 ± 1.61 (2)	5.2 ± 0.38	6.8 ± 0.43

[a] Log_{10} geometric mean colony count per gram of tissue ± standard error obtained for three chickens. Groups of 21 chicks were inoculated when they were 7 days old with *S. enterica* serovar Typhimurium χ8879 (Δ*sptP1033*::*xylE* Δ*asdA16* Δ*phoP233*) harboring plasmid pYA3870 or pYA4157 or with *S. enterica* serovar Typhimurium χ9242 (Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT) harboring pYA4184.
[b] The numbers in parentheses are the numbers of chicks with less than 10 CFU/g of tissue.

serum antibody titers were identical for all groups of chickens (Fig. 5). Three weeks after the second immunization (when the animals were 5 weeks old), there were no differences in serum antibody titers between unimmunized chickens (group G1) and chickens that were immunized with RASV strains containing vector controls (group G2). Consistent with previous observations (unpublished data), delivery of the antigen via the T3SS alone did not induce an appreciable level of antigen-specific antibodies (group G3), while higher antibody titers to SO7 were obtained for group G4, which received the primary immunization via the T3SS and the secondary immunization via the T2SS (Fig. 5). The highest titers of anti-SO7 antibodies were obtained for groups G5 and G6, which were either primed with a T2SS-SO7 construct and boosted with a T3SS-SO7 construct (group G5) or primed and boosted with a T2SS-SO7 construct (group G6).

Challenge of chickens with *Eimeria* and protection by RASV strains. Chickens challenged with *E. acervulina* were immunized with RASV strains delivering the SO7 antigen and an RASV strain expressing *E. acervulina* merozoite antigen EAMZ250 fused to the translocation domain of the effector protein SptP (20). The smallest body weight gains were observed for the control group, in which chicks were immunized with RASV strains harboring antigen-free vectors (group G2) (Table 3). All groups that were immunized with RASV strains harboring *Eimeria* antigens (groups G4 to G6) had greater body weight gains than the control group that was immunized with *Salmonella* vector control (group G2). Chickens immunized with RASV strains expressing EAMZ250 and the SO7 antigen via the T3SS (group G3) gained significantly more weight than the control (group G2) ($P < 0.05$) (Table 3). In addition, the weight gain for this group was similar to the weight gain for the unchallenged controls (group G1), indicating that there was significant protection against challenge ($P < 0.05$). No differences in feed conversion ratios were observed among groups (Table 3). These results suggest that induction of cell-mediated immune responses to multiple antigens (EAMZ250 and SO7) leads to superior protection against challenge by *E. acervulina*.

In the second challenge study all groups were immunized

with RASV strains expressing the SO7 antigen (groups G3 to G5), and the animals gained significantly more weight than the challenged control animals (group G2) ($P < 0.05$) (Table 4). Immunization with an RASV strain that delivered the SO7 antigen via the T2SS (group G5) appeared to confer the most protection, as the body weight gain for chicks in this group was not statistically different from the body weight gain for chicks in the unchallenged control (group G1) ($P > 0.05$) (Table 4). In addition, groups that were immunized with SO7-expressing RASV strains (groups G4 and G5) exhibited significantly improved feed utilization compared to the challenged control (group G2) ($P < 0.05$) (Table 4). Again, group 5, which exhibited the greatest weight gain, had a feed conversion ratio that was not statistically different from that of the unchallenged control (group G1) ($P < 0.05$) (Table 4). These data suggest that immune responses elicited to the SO7 antigen delivered via RASV strains provide various degrees of protection against a challenge with pathogenic *E. tenella*, and immunization with T2SS constructs appears to be the most protective type of immunization.

DISCUSSION

In spite of the advances that have been made in drug and vaccine development, coccidiosis remains one of the leading causes of morbidity and mortality for poultry. The economic losses of poultry producers due to this parasite worldwide have been estimated to range from $800 million to $1.5 billion per year (15, 36, 39). Development of a protective vaccine against this disease, although very challenging, is a desirable approach for decreasing its negative economic impact and for improving the welfare of poultry. A number of RASV strains have been developed and are able to bear and express several *Eimeria* antigens for induction of antigen-specific immune responses that ultimately lead to protection against challenge by this parasite (20, 27). Thus far, none of these antigens have been found to fully protect against coccidiosis. RASV strains are an
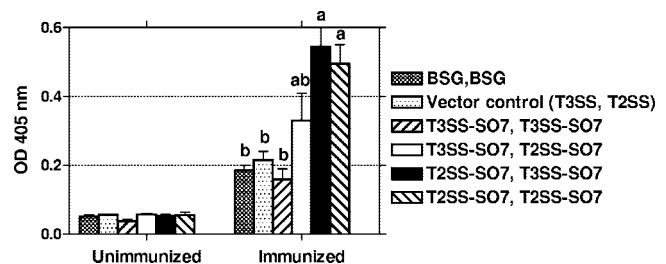

FIG. 5. Antibody titers against the SO7 antigen in sera of unimmunized and RASV strain-immunized chickens. Serum was collected from chickens prior to immunization (Unimmunized) and 3 weeks after secondary immunization with RASV strains (Immunized). Individual sera of five chicks per group were diluted 1:250, and IgG antibody titers were determined by an ELISA. The immunization groups are indicated as follows: group G1, BSG, BSG; group G2, Vector control (T3SS, T2SS); group G3, T3SS-SO7, T3SS-SO7; group G4, T3SS-SO7, T2SS-SO7; group G5, T2SS-SO7, T3SS-SO7; and group G6, T2SS-SO7, T2SS-SO7. The bars indicate means, and the error bars indicate standard deviations. The letters above the bars indicate the significance of differences for the means; means that do not share a letter are significantly different ($P < 0.05$). OD 405 nm, optical density at 405 nm.

TABLE 3. Immunization of chickens with RASV strains and *E. acervulina* oocyst challenge

Group	Primary immunization[a]	Secondary immunization	Oocyst challenge[b]	Weight gain (g) (mean ± SEM)[c]	Feed conversion ratio[c]
G1	BSG	BSG	No	141 ± 7 A[d]	2.93 A
G2	χ8879(pYA3870) (T3SS-vector control)	χ9242(pYA3493) (T2SS-vector control)	Yes	127 ± 2 B	2.94 A
G3	χ8879(pYA3658) (T3SS-EAMZ250)	χ8879(pYA4157) (T3SS-SO7)	Yes	140 ± 3 A	2.75 A
G4	χ8879(pYA3658) (T3SS-EAMZ250)	χ9242(pYA4184) (T2SS-SO7)	Yes	136 ± 5 AB	2.76 A
G5	χ8879(pYA4157) (T3SS-SO7)	χ9242(pYA4184) (T2SS-SO7)	Yes	132 ± 1 AB	2.71 A
G6	χ9242(pYA4184) (T2SS-SO7)	χ9242(pYA4184) (T2SS-SO7)	Yes	135 ± 1 AB	2.85 A

[a] Chickens received a primary immunization when they were 1 week old and a secondary immunization 1 week later. Strains χ8879 (Δ*sptP1033::xylE* Δ*asdA16* Δ*phoP233*) and χ9242 (Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P$_{BAD}$ *lacI* TT) were used for immunization.

[b] Three weeks after the secondary immunization, chickens in groups G2 to G6 were inoculated with 10^5 *E. acervulina* oocysts.

[c] Right before the oocyst challenge and on day 6 postchallenge, the body weights were recorded, and the weight gain and feed conversion ratio (grams of feed consumed per gram of body weight gained) were calculated.

[d] Means that are not followed by the same letter are significantly different ($P < 0.05$).

attractive approach since they are safe, are easy to administer, are cost-effective, and do not contain antibiotic resistance genes. Previously, we reported that the EAMZ250 antigen of *E. acervulina* delivered via the T3SS of an RASV strain provided partial protection against *E. acervulina* challenge, while the EASZ240 antigen delivered in a similar way did not (20). This finding further emphasizes the importance of the antigen that is used for the efficacy of an RASV strain; therefore, both EAMZ250 (20) and the SO7 antigen were delivered via the T3SS and T2SS of *Salmonella*.

SO7 is a sporozoite antigen of *E. tenella* and was shown to protect antigen-inoculated chickens against infectious challenge with four different species of *Eimeria* (6). However, intramuscular injection of purified antigen into each chicken as a form of immunization is impractical, time-consuming, and costly. Although this type of parenteral immunization favors systemic humoral immune responses rather than cell-mediated immune responses (25), no SO7-specific antibodies were detected in immunized chicks (6). The protection against challenge in these studies was attributed to cell-mediated immune responses, since the SO7 antigen is a class B antigen (19). Other workers have found that when injected subcutaneously, SO7 induces strong systemic IgG responses and lower, but detectable, IgA responses, indicating that SO7 is immunogenic (27). In addition, IgG titers in serum and IgA titers in bile were observed when this antigen was delivered via attenuated *Salmonella* strains (27); however, since no challenge experiments were conducted with *Salmonella*-immunized chickens, it is dif-

ficult to ascertain the relevance of this immunity for protection against challenge with *Eimeria* parasites.

For delivery of *Eimeria* antigens into host cell cytoplasm, translocation domains of the effector proteins SptP and SopE of *Salmonella* are being used. Although similar amounts of the SptP and SopE effector proteins are injected by *Salmonella* into the cytoplasm of eukaryotic cells, SopE is rapidly processed via the ubiquitin-mediated proteosome degradation pathway, while SptP is processed at a much lower rate (21). In addition, the secretion and translocation signals of SopE were shown to be more efficient for delivering various Gag constructs to the class I antigen-presenting pathway than the signals derived from SptP were (5); therefore, for delivery of the SO7 antigen via the T3SS we used the 80-amino-acid translocation domain of the SopE protein.

We found that RASV strains χ8879 and χ9085 specify synthesis of the SopE-SO7 fusion protein and secrete it into the culture supernatant via the T3SS. However, SO7 is toxic to both *E. coli* and *Salmonella* when it is expressed at higher levels by medium-copy-number (pBR *ori*) plasmids, which has not been reported previously. The reason for this could be that other authors expressed SO7 as a fusion with a TetC fragment, which stabilizes its expression (27). To overcome the toxicity issues, we devised an antigen delivery approach in which the expression of the SO7 antigen in vitro is suppressed by supplemental arabinose in the medium. The expression of the antigen should commence in vivo only after *Salmonella* has depleted its intracellular arabinose and has colonized the lym-

TABLE 4. Immunization of chickens with RASV strains and *E. tenella* oocyst challenge

Group	Primary immunization[a]	Secondary immunization	Oocyst challenge[b]	Weight gain (mean ± SEM)[c]	Feed conversion ratio[c]
G1	BSG	BSG	No	141 ± 7 A[d]	2.93 C
G2	χ8879(pYA3870) (T3SS-vector control)	χ9242(pYA3493) (T2SS-vector control)	Yes	92 ± 11 D	4.12 A
G3	χ8879(pYA4157) (T3SS-SO7)	χ8879(pYA4157) (T3SS-SO7)	Yes	102 ± 4 BC	3.71 AB
G4	χ8879(pYA4157) (T3SS-SO7)	χ9242(pYA4184) (T2SS-SO7)	Yes	116 ± 12 BC	3.12 BC
G5	χ9242(pYA4184) (T2SS-SO7)	χ9242(pYA4184) (T2SS-SO7)	Yes	124 ± 6 AB	3.14 BC

[a] Chickens received a primary immunization when they were 1 week old and a secondary immunization 1 week later. Strains χ8879 (Δ*sptP1033::xylE* Δ*asdA16* Δ*phoP233*) and χ9242 (Δ*phoP233* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P$_{BAD}$ *lacI* TT) were used for immunization.

[b] Three weeks after the secondary immunization, chickens in groups G2 to G6 were inoculated with 10^5 *E. tenella* oocysts.

[c] Right before the oocyst challenge and on day 6 postchallenge, body weights were recorded, and the weight gain and feed conversion ratio (grams of feed consumed per gram of body weight gained) were calculated.

[d] Means that are not followed by the same letter are significantly different ($P < 0.05$).

phoid tissues, where it interacts with cells of the lymphoid system.

Except for a short period of time, all developmental stages of *Eimeria* occur within intestinal tissues; thus, inducing mucosal immunity to this parasite is critical. One important feature of RASV strains is their ability to colonize and persist in mucosa and other lymphoid tissues and induce immune responses without causing disease symptoms. In this study we demonstrated that RASV strains colonize and persist in the cecum, bursa, and spleen of 1-week-old non-SPF chicks with an established intestinal microflora, although the titers of the RASV strains were lower than the titers of RASV strains observed in tissues of SPF chickens (20). *Salmonella*-specific immune responses generated after administration of the first dose of RASV strains could further diminish the ability of RASV strains to colonize tissues; therefore, the birds were immunized with RASV strains a second time just 6 days after the first immunization.

To diversify the repertoire of the immune responses and thus provide better protection against challenge, the SO7 and EAMZ250 antigens were delivered via the T3SS and T2SS of *Salmonella*. Antigen delivery via the T3SS induces mucosal cytotoxic T-cell responses to heterologous antigens (8), while delivery of antigens via the T2SS induces both Th1- and Th2-mediated immune responses in mice (17), which are characterized by high antibody titers to heterologous antigens. Due to the inherent variability in T-cell responses in outbred populations and due to the lack of reagents available, we did not determine SO7-specific T-cell responses in RASV strain-immunized chickens. Moreover, although the SO7 antigen is a class B antigen, some chickens do not respond to it (6). Therefore, we used protection against challenge and serum antibody titers as indicators of the immunogenicity of RASV strains harboring the SO7 antigen.

Although *Eimeria*-specific antibodies may not be important for protection against challenge, one study was conducted in which anti-SO7 antibody titers in serum of RASV strain-immunized chickens were determined. The presence of anti-SO7 antibodies was an indication that the antigen was delivered and that prime-boost immunization with various strains and constructs was effective. The lack of antigen-specific antibody titers in sera of chickens immunized twice with T3SS constructs (group G3) is in agreement with our previous finding (unpublished observations) that delivery of heterologous antigen by this route does not yield an appreciable amount of heterologous antigen-specific antibodies (group G3). However, when chickens were immunized with a T3SS construct followed by a T2SS construct (group G4), the antibody titers to the heterologous antigen increased appreciably. It is interesting that the highest antibody titers were obtained for sera of chickens that were primed with a T2SS construct (groups G5 and G6). The lack of differences between these two groups indicates that boosting with T3SS (group G5) did not further increase the antigen-specific titers (as expected); however, it would be interesting to investigate whether cell-mediated immune responses are enhanced using this vaccination strategy. A second immunization with a T2SS construct (group G6) did not increase the antibody titers additionally, suggesting that a single immunization dose of 10^9 CFU of an RASV strain is sufficient

for induction of maximal antibody titers to the heterologous antigen.

Several criteria, such as oocyst production, the presence of lesions in the intestines, body weight gain, and feed conversion efficiency, have been used to evaluate the efficacy of *Eimeria* vaccines (4, 40), but the most useful criterion is undoubtedly the weight gain of vaccinated birds following a challenge with *Eimeria* (4, 40). While the presence of intestinal lesions and oocyst output can be measured, these parameters should not be evaluated in isolation but should be correlated with other more reliable criteria, especially the growth rate (3, 4). *Eimeria* spp. are single-cell intestinal protozoan parasites that probably do not share antigens with *Salmonella*; therefore, immunization with *Salmonella* alone should not induce *Eimeria*-specific immune responses. However, it is possible that innate and acquired immune responses induced by *Salmonella*, although nonspecific, could provide some protection against challenge by *Eimeria*. Thus, in both studies we used a control group in which chickens were immunized with RASV strains harboring the vector control (group G2) (Tables 3 and 4) and challenged with *Eimeria* oocysts. This control accounted for any protection (immunity) due to the *Salmonella* vector alone and highlighted the protection provided by the *Eimeria* SO7 and EAMZ250 antigens delivered via *Salmonella* (groups G3 to G6) (Tables 3 and 4). In several previous studies we used two different controls: animals immunized with BSG (mock immunized) and then challenged with *Eimeria* and animals immunized with *Salmonella* harboring control vectors (without *Eimeria* antigens) and then challenged with *Eimeria*. In all studies we observed no significant differences in lesion scores or weight gain (indices of protection against challenge) between these two control groups (20; unpublished data). The lack of differences between these two controls and the lack of protection in group G2 (immunized with an RASV strain harboring a vector control) in the present work indicate that immunization with *Salmonella* alone does not induce significant protection against challenge with *Eimeria*.

Immunization with either the SO7 or EAMZ250 antigen led to some improvement in body weight gain; however, delivering both antigens via the T3SS appears to be the most efficient immunization strategy. EAMZ250 and SO7 may contain several protective epitopes, and induction of CD8$^+$ T-cell-mediated immune responses (which antigen delivery via T3SS favors) to multiple epitopes leads to better protection against challenge.

We observed more dramatic results with groups of chickens that were challenged with *E. tenella*, including significantly better weight gain and feed conversion in all SO7-immunized *E. tenella*-challenged chickens. In contrast to the results for *E. acervulina* challenge, it appears that the most efficient immunization strategy for *E. tenella* is delivery of the antigen via the T2SS, suggesting that humoral immune responses in combination with cell-mediated responses might aid in protection against challenge, as appeared to be the case in the *E. tenella* challenge study. It is possible that immunity to *E. acervulina* and immunity to *E. tenella* occur by different mechanisms because these organisms invade different regions of the gut.

In conclusion, RASV strains are able to colonize the lymphoid tissues of 1- and 2-week-old chickens, induce antigen-specific humoral immune responses, and provide protection

against challenge with *E. acervulina* and *E. tenella*. Using a combination of the T3SS and T2SS of *Salmonella* in a prime-boost immunization strategy might be an optimal approach for induction of both cell-mediated and humoral immune responses to protective antigens and ultimately provide protection against challenge with an infectious parasite. The progress made thus far in improving the design of RASV strains and antigen delivery strategies, coupled with identification of novel protective antigens from different species of *Eimeria*, should help workers develop efficacious vaccines against these parasites.

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Editor: A. J. Bäumler